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The *HRPT2* Tumor Suppressor Gene Product Parafibromin Associates with Human PAF1 and RNA Polymerase II

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Inactivation of the *HRPT2* tumor suppressor gene is associated with the pathogenesis of the hereditary hyperparathyroidism-jaw tumor syndrome and malignancy in sporadic parathyroid tumors. The cellular function of the *HPRT2* gene product, parafibromin, has not been defined yet. Here we show that parafibromin physically interacts with human orthologs of yeast Paf1 complex components, including PAF1, LEO1, and CTR9, that are involved in transcription elongation and 3′ end processing. It also associates with modified forms of the large subunit of RNA polymerase II, in particular those phosphorylated on serine 5 or 2 within the carboxy-terminal domain, that are important for the coordinate recruitment of transcription elongation and RNA processing machineries during the transcription cycle. These interactions depend on a C-terminal domain of parafibromin, which is deleted in ca. 80% of clinically relevant mutations. Finally, RNAi-induced downregulation of parafibromin promotes entry into S phase, implying a role for parafibromin as an inhibitor of cell cycle progression. Taken together, these findings link the tumor suppressor parafibromin to the transcription elongation and RNA processing pathway as a PAF1 complex- and RNA polymerase II-bound protein. Dysfunction of this pathway may be a general phenomenon in the majority of cases of hereditary parathyroid cancer.

Primary hyperparathyroidism (HPT) is one of the most common endocrinopathies characterized by the formation of parathyroid tumors. Parathyroid lesions are mainly benign, diagnosed as adenoma or hyperplasia in >95% of the cases. However, 1 to 5% of HPT patients develop parathyroid carcinoma, which are associated with major morbidity and mortality (18, 30). The majority of these tumors are sporadic, but 5% are associated with the autosomal-dominant hereditary cancer syndromes multiple endocrine neoplasia type 1 (MEN1) and MEN2A, familial hyperparathyroidism (FIHP), and hyperparathyroidism-jaw tumor (HPT-JT) syndrome (9, 17, 29, 39).

HPT-JT syndrome is characterized by parathyroid tumors, fibro-osseous lesions of the mandible and maxilla, as well as renal cysts and tumors (8). It is noteworthy that, whereas parathyroid carcinoma are rare in sporadic disorders, their occurrence increases to ca. 15% in patients with HPT-JT (19, 35). Recently, the gene whose inactivation is directly associated with the pathogenesis of the HPT-JT syndrome has been identified as the *HRPT2* tumor suppressor gene (3). In fact, nearly all mutations reported thus far are predicted to be inactivating mutations, and *HRPT2*-linked families show loss of heterozygosity at the relevant locus with retention of the mutant allele. Therefore, the behavior of this gene follows Knudson's "two-hit" hypothesis of tumor suppressor genetics. Mutations in *HRPT2* have also been found in sporadic parathyroid carcinoma, as well as in a subset of FIHP, and are strongly associated with tumor malignancy (6, 31, 38). Thus, *HRPT2* tumor suppressor gene inactivation is a critical event in the development of both hereditary and sporadic parathyroid cancers.

The *HRPT2* gene is ubiquitously expressed and encodes a predicted protein of 531 amino acids, termed parafibromin (3). The primary sequence of parafibromin neither closely resembles other known proteins nor reveals obvious structural motifs that might provide a direct clue as to its function. The exception is an ~200-amino-acid C-terminal segment of parafibromin, which displays modest homology (27%) to budding yeast Cdc73, a component of the Paf1 complex that functions at various stages during the yeast transcription cycle. Obviously, this licenses the speculation that parafibromin might function in a similar pathway in human cells.

The Paf1 complex has been originally identified as an RNA polymerase II (RNAP II)-associated complex (32, 33) and minimally contains Paf1, Cdc73, Rtf1, Leo1, and Ctr9 (20). It has been implicated in the regulation of genes whose products function in metabolism and cell cycle control (2, 26). Genetic and biochemical evidence in yeast suggest key roles for Paf1 complex components at various stages of the gene expression pathway, including transcript site selection (34), transcriptional elongation (25, 27, 33), histone H2B monoubiquitination and subsequent histone H3 methylation (13, 22, 23, 40), and more recently poly(A) length control and the coupling of transcriptional and posttranscriptional events (21).

The function of the Paf1 complex has also been intimately linked to site-specific phosphorylation events of RNAP II within its carboxy-terminal domain (CTD) (24). Site-specific phosphorylation of RNAP II CTD is an important mechanism that contributes, at least in part, to the normal temporal coordination of the activities of the various protein assemblages involved in mRNA synthesis. For example, during the transi-

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tion from transcription initiation to elongation, serine 5 of CTD is phosphorylated. As RNAP II elongates, serine 5 phosphorylation diminishes while serine 2 phosphorylation increases. The latter initiates the recruitment of factors involved in subsequent steps of RNA processing (1, 10).

We have recently reported on the identification and functional characterization of a new multiprotein complex in human cells whose central component is URI, an unconventional member of the prefoldin (PFD) family of ATP-independent molecular chaperones (4). URI is believed to function as a scaffolding protein that is able to assemble through its dedicated PFD- and RBP5-binding domains additional members of the PFD family, as well as three proteins with key roles in transcription. These include the RNAP II core subunit RPB5 and the ATPases TIP48 and TIP49, which are also present in various chromatin remodeling complexes. Initial functional characterization of URI in yeast and human cells has pointed to a role for this protein in gene expression controlled by TOR (for target of rapamycin), a phosphoinositide-3-kinase-related protein kinase that integrates nutritional cues into a coherent cell growth and proliferative response (7). Here we report on the identification of novel URI-associated proteins including the tumor suppressor parafibromin and human orthologs of the yeast Paf1 complex. Our studies also reveal an association of parafibromin with the serine 5- and serine 2-phosphorylated forms of RNAP II CTD and demonstrate that a naturally occurring tumor-derived mutant of parafibromin lacks PAF1 and RNAP II binding function. These data infer a potential role of the tumor suppressor parafibromin in transcriptional/posttranscriptional control pathways.

MATERIALS AND METHODS

Purification of URI-associated proteins by mass spectrometry and gel filtration analysis. Forty 10-cm dishes of 293 cells were lysed for 30 min on ice in 80 ml of TNN buffer (50 mM Tris-HCl [pH 7.5], 250 mM NaCl, 5 mM EDTA, 0.5% NP-40, 50 mM NaF, 0.2 mM Na_3VO_4, 1 mM dithiothreitol [DTT], 1 mM phenylmethylsulfonyl fluoride [PMSF], 10 μg of aprotinin/ml) and then Dounce homogenized (15 strokes with a B pestle), and lysates were cleared by centrifugation at 18,000 × *g* for 10 min. Supernatants were collected and sequentially incubated first with control mouse immunoglobulin G (IgG) and then with monoclonal anti-URI antibodies (a mix of monoclonal antibodies [MAbs] 179.2.1, 179.22.34, and 179.58.2), both covalently coupled to protein A/G-Sepharose by using the dimethylpimelimidate coupling procedure. Immunobeads from both incubations were washed four times with TNN buffer, and proteins were eluted with 300 μl of 0.2 M glycine-HCl (pH 2.5), neutralized with 100 μl of 1 M Tris-HCl (pH 7.5), and precipitated and resolved on a 6 to 12% gradient sodium dodecyl sulfate (SDS)-polyacrylamide gel. The gel was stained with colloidal blue, and the protein bands were excised, digested with trypsin, and subjected to sequence analysis. Peptides were sequenced by using nano-electrospray tandem mass spectrometry on an API 300 mass spectrometer (PE Sciex, Toronto, Ontario, Canada) and identified as described previously (16). Gel filtration was done exactly as previously described (4).

Plasmid constructions. The commercially available cDNA IMAGE clones 592083 and 3504227 encoding full-length human parafibromin and PAF1 homologs, respectively, were obtained from the Human Genome Mapping Project-Resource Centre. For bacterial expression, full-length PAF1 and parafibromin cDNAs were subcloned by one-step PCR into pGEX-2TK and pGEX-4T1, respectively [constructs are referred to as pGEX-PAF1 and pGEX-parafibromin(wt)]. To express the C-terminal segment of parafibromin as a glutathione *S*-transferase (GST) fusion protein [parafibromin(378-531)], the sequence from nucleotides 1135 to 1593 was amplified with the appropriate primers and subcloned into pGEX-4T1 [construct pGEX-parafibromin(378-531)]. For transient expression into mammalian cells, full-length PAF1 and parafibromin cDNAs were subcloned by one-step PCR into pcDNA3-HA to generate pcDNA3-HA-PAF1 and pcDNA3-HA-parafibromin(wt). Truncated forms of parafibromin's cDNA, encoding the mutants parafibromin(R222X) or parafibromin(200-531), were generated by PCR amplification of the sequence from nucleotides 1 to 664 and nucleotides 600 to 1593, respectively, with appropriate primers and subcloned into pGEX-4T1 and/or pcDNA3-HA to create pGEX-parafibromin (R222X), pcDNA3-HA-parafibromin(R222X), pcDNA3-HA-parafibromin(200-531), and pcDNA3-HA-parafibromin(NLS-200-531), respectively. Details of the generation of constructs are available upon request.

Antibodies. Rabbit polyclonal anti-parafibromin(cp) [anti-parafibromin(cp)] and anti-human PAF1(cp) [anti-PAF1(cp)] antibodies were raised against 20-mer synthetic peptides corresponding to their respective C termini (parafibromin peptide, CRFWETLDRYMVKHKSHLRF; PAF1 peptide, CEDGSEAAASDS SEADSDSD). Anti-peptide antibodies were affinity purified as described previously (15). Rabbit polyclonal antibody specific for the C-terminal half of parafibromin (amino acids 378 to 531) [anti-parafibromin(cfp)] was raised against bacterially produced GST fusion protein. The serum was affinity purified by incubation first with a GST column, followed by a GST-parafibromin(378-531) fusion protein column prepared by covalently cross-linking the respective proteins to glutathione-Sepharose beads with dimethylpimelimidate (5). Polyclonal anti-LEO1 and anti-CTR9 antibodies were from Bethyl Laboratories, Inc. Mouse MAb H14 directed against phospho-Ser5 CTD RNAP II has been described previously (37). Mouse MAbs 4H8 and H5 directed against CTD RNAP II and phospho-Ser2 CTD RNAP II were purchased from Abcam and Covance, respectively. MAbs directed against URI 179.63.2, 179.2.1, 179.22.34, and 179.58.2 have been previously described (4). Monoclonal anti-hemagglutinin (HA) antibody (clone 12CA5) was purchased from Boehringer Mannheim.

Immunoprecipitation and immunoblotting. For immunoprecipitation experiments, one 10-cm dish of HeLa cells was lysed in 1 ml of ice-cold lysis buffer (20 mM Tris-HCl [pH 7.4], 150 mM NaCl, 1 mM EDTA, 10% glycerol, 1% NP-40, 10 μg of aprotinin/ml, 1 mM PMSF, 50 mM NaF, 1 mM Na_3VO_4, 1 mM DTT). After shaking for 15 min at 4°C, whole-cell extracts were centrifuged at 4°C for 15 min at top speed in an Eppendorf microcentrifuge, and supernatants were preincubated for 30 min at 4°C with 25 μl of a 50% (wt/wt) slurry of protein A-Sepharose (Pharmacia), followed by centrifugation. Supernatants were incubated with 3 to 5 μg of the indicated antibody for 2 h at 4°C. Immunocomplexes were then collected with protein A-Sepharose for 1 h and washed three times with lysis buffer. To prepare whole-cell extracts for immunoblot analysis, cells were lysed in 500 μl of lysis buffer containing 20 mM HEPES (pH 8.0), 0.4 M NaCl, 25% glycerol, 1 mM EDTA, 15 mM NaF, 0.1% NP-40, 2.5 mM DTT, 0.5 mM Na_3VO_4, 1 μg of aprotinin/ml, and 0.5 mM PMSF. Extracts were incubated on ice for 30 min and centrifuged at 4°C for 15 min at top speed. Immunoblotting was performed as previously described.

GST pull-down experiments. Bacteria transformed with pGEX, pGEX PAF1, pGEX-parafibromin(wt), or pGEX-parafibromin(R222X) were grown overnight in LB medium supplemented with 100 μg of ampicillin/ml. After 3 h of induction with 0.1 mM IPTG (isopropyl-β-D-thiogalactopyranoside), bacteria were collected for 5 min at 5,000 × *g*. The pellet was lysed in NET-N buffer (20 mM Tris [pH 8.0], 150 mM NaCl, 1 mM EDTA, 0.5% NP-40, 1 mM PMSF, 1 mM DTT, 1 μg of aprotinin/ml), sonicated, and centrifuged at 10,000 × *g* for 10 min. Supernatant was incubated with glutathione-Sepharose beads for 1 h at 4°C and then beads were washed four times with lysis buffer. In parallel, in vitro-translated HA-PAF1, HA-parafibromin(wt), and HA-parafibromin(R222X) were produced by using TNT-coupled reticulocyte lysate system (Promega) according to the manufacturer's procedure. GST pull-downs were performed by incubating 2 μg of the indicated GST-fusion protein with 5 μl of the indicated in vitro translation mix in 500 μl of TNN buffer for 1 h at 4°C. Beads were washed four times in TNN buffer, and then the proteins were resolved by SDS-polyacrylamide gel electrophoresis (PAGE) and detected by fluorography.

Cell culture, transfection, and cell cycle analysis. HeLa, HEK-293, and U2-OS cells were cultivated in Dulbecco modified Eagle medium supplemented with 10% fetal calf serum (Gibco) at 37°C. For serum starvation experiments, U2-OS cells at 50% confluency were maintained for 48 h in Dulbecco modified Eagle medium. Transfection of small interfering RNA (siRNA; target sequences 5′-A ACTGCCCTTAAACAGAGGAG-3′ for parafibromin and 5′-AAGCAGCAGT TTACCGAGGAA-3′ for PAF1) was performed by using OligoFectamine (Invitrogen) as the transfection reagent according to the instructions of the manufacturer. Cells were harvested 48 h after transfection, unless otherwise indicated. For biochemical analyses, 50% confluent HeLa cells were transiently transfected by using the $CaCl_2$ method as previously described (11) with 10 μg of plasmid DNA and harvested ~24 h after removal of the precipitate. For cell cycle analysis, HeLa or U2-OS cells were harvested by trypsinization and processed for flow cytometric analysis as described previously (12).

Immunofluorescence microscopy. HeLa cells were plated in 35-mm dishes containing coverslips and processed for indirect immunofluorescence as previ-

ously described (15). Briefly, cells were washed with phosphate-buffered saline (PBS), fixed for 30 min in 3.7% paraformaldehyde at 37°C, rinsed quickly three times with PBS, incubated for 5 min with 0.2% Triton X-100 in PBS, and washed again quickly three times with PBS. Coverslips were incubated with 5 μg of anti-parafibromin(cfp) antibody/ml, preadsorbed as indicated on glutathione-Sepharose beads coupled to 1 μg of GST-parafibromin(378-531) fusion protein, in PBS supplemented with 5% bovine serum albumin and 0.2% goat serum for 1 h. After three washes in PBS, fluorescein isothiocyanate-conjugated anti-rabbit antibody (Jackson Immunoresearch, Inc.) was applied, together with 1 μg of DAPI (4′,6′-diamidino-2-phenylindole; Sigma)/ml for 1 h. Cells were washed as described above, mounted in Vectashield, and viewed with a Zeiss fluorescence microscope. For detection of HA-tagged proteins, cells were first transfected by using FuGENE reagent (Roche) with 1 μg of the indicated plasmids according to manufacturer's procedure. At 24 h posttransfection, cells were processed as described above, with anti-HA MAb as primary antibody and FITC-conjugated anti-mouse (Jackson Immunoresearch, Inc.) as secondary antibody.

RESULTS

Identification of novel URI-associated proteins. To further define protein complexes containing endogenous URI, we purified URI-associated proteins from unfractionated HEK-293 cell extracts by one-step immunoprecipitation with a mix of three URI MAbs covalently coupled to protein A/G-Sepharose. After extensive washing, proteins bound to the beads were eluted under native conditions, electrophoresed on a polyacrylamide-SDS gel, and colloidal blue stained. A number of proteins were specifically enriched in URI immunoprecipitates but not in control immunoprecipitates performed in parallel (Fig. 1A, compare lanes 3 and 2). The relevant bands were excised from the gel and processed for tryptic digestion within the gel material. The digest was eluted and subjected to tandem mass spectrometric analysis, which provided a rich harvest of URI-associated proteins. Interestingly, in addition to the known URI-associated proteins reported previously (4), we identified additional partner proteins of URI.

Of particular interest were four proteins with apparent molecular sizes of 70, 90, 110, and 140 kDa. The 70-kDa species is parafibromin, the product of the *HRPT2* tumor suppressor gene. The 90-kDa protein band contained, in addition to URI, the human homolog of *Saccharomyces cerevisiae* Paf1. The 110- and 140-kDa proteins are the human orthologs of *S. cerevisiae* Leo1 and Ctr9, respectively. Thus, URI associates with human orthologs of yeast Paf1 complex components and the parafibromin tumor suppressor. In this regard, previous database searches revealed a limited homology of 27% between parafibromin and yeast Cdc73 (3). This observation combined with the presence of human PAF1, LEO1, and CTR9 in URI immunoprecipitates raised the interesting possibility that parafibromin might, despite moderate sequence homology, be the human counterpart of yeast Cdc73.

To further explore this, we raised antibodies to a synthetic 20mer peptide corresponding to the predicted C terminus of parafibromin [anti-parafibromin(cp)] and to a 200-amino-acid C-terminal fragment of parafibromin expressed as a GST fusion protein in bacteria [anti-parafibromin(cfp)]. We also raised antibodies to a synthetic 20mer peptide corresponding to the predicted C terminus of PAF1 [anti-PAF1(cp)]. As shown in Fig. 1B, on Superose 6 gel filtration, parafibromin eluted as a single, moderately broad peak of <1 MDa. In addition, we observed substantial overlap between fractions that contained parafibromin and those harboring URI. Interestingly, the fractions 10 to 17, in which parafibromin and URI coeluted, were also enriched for PAF1, LEO1, and CTR9, supporting the view that these components coexist in a biochemical complex. It is noteworthy that there was little overlap between the RTF1 and URI elution profiles, a finding consistent with the observation that RTF1 was not detected by mass spectrometry in URI immunoprecipitates. Moreover, the RTF1 peak did not intersect with the PAF1- or CTR9-enriched fractions but overlapped with some of the fractions harboring parafibromin and LEO1. Taken together, these data suggest that the tumor suppressor parafibromin is a component of a human PAF1 complex.

Biochemical evidence of an in vivo interaction of parafibromin with PAF1 complex components. To verify the interaction between URI, parafibromin, and components of the human PAF1 complex, we performed reciprocal coimmunoprecipitation experiments. As shown in Fig. 2A, HEK-293 and HeLa cell immunoprecipitates generated with the URI MAb mix contained endogenous parafibromin and hPAF1, as determined by immunoblotting (lanes 1 and 3, respectively). We also found LEO1 and CTR9 in URI immunoprecipitates (data not shown). None of these proteins were detected in immunoprecipitates with control mouse IgG (Fig. 2A, lanes 2 and 4). PAF1, LEO1, and CTR9 were also readily detected in anti-parafibromin(cfp) immunoprecipitates (Fig. 2B, lane 1) but not in the corresponding control precipitates (Fig. 2B, lanes 2 and 3). Finally, parafibromin, LEO1, CTR9, and URI were also coimmunoprecipitated with anti-PAF1(cp) antibody (Fig. 2C, lane 1) but not with peptide-blocked antibody (Fig. 2C, lane 2) or control rabbit IgG (Fig. 2C, lane 3). These data confirm that the tumor suppressor parafibromin forms stable complexes with URI and the PAF1 complex components PAF1, LEO1, and CTR9 in vivo.

Parafibromin and PAF1 interact with the RNAP II large subunit. In yeast, Paf1 and Cdc73 have been among the first factors shown to associate with RNAP II in a form that is biochemically distinct from the Srb/Mediator complex (32). Given this and the fact that parafibromin interacts with the human PAF1 complex, we sought to determine whether endogenous parafibromin and/or PAF1 would interact with RNAP II in vivo. To this end, HeLa cells extracts were subjected to immunoprecipitation with anti-RNAP II MAb 4H8. As shown in Fig. 3A, whereas a control mouse MAb failed to immunoprecipitate parafibromin or PAF1 (lane 2), MAb 4H8 immunoprecipitates contained both parafibromin and PAF1 (lane 1, first and second panels). Importantly, URI was also found in RNAP II immunoprecipitates (lane 1, third panel), a finding consistent with the notion that URI is part of a parafibromin-PAF1 complex and with earlier results that revealed interaction between URI and the RNAP II core subunit RPB5 (4). In reciprocal experiments, anti-parafibromin(cfp) and anti-PAF1(cp) immunoprecipitations contained RNAP II (Fig. 3B, upper and lower panels, respectively). These results suggest that the parafibromin tumor suppressor and two of its partner proteins, PAF1 and URI, interact with RNAP II.

During its transit from transcription initiation to elongation and termination, RNAP II associates with an extraordinary array of proteins; this is, in part, controlled by phosphorylation of the RNAP II CTD at distinct sites (24). During the transition from transcription initiation to elongation serine 5 of the CTD is phosphorylated. Later, the serine 2 phosphorylated

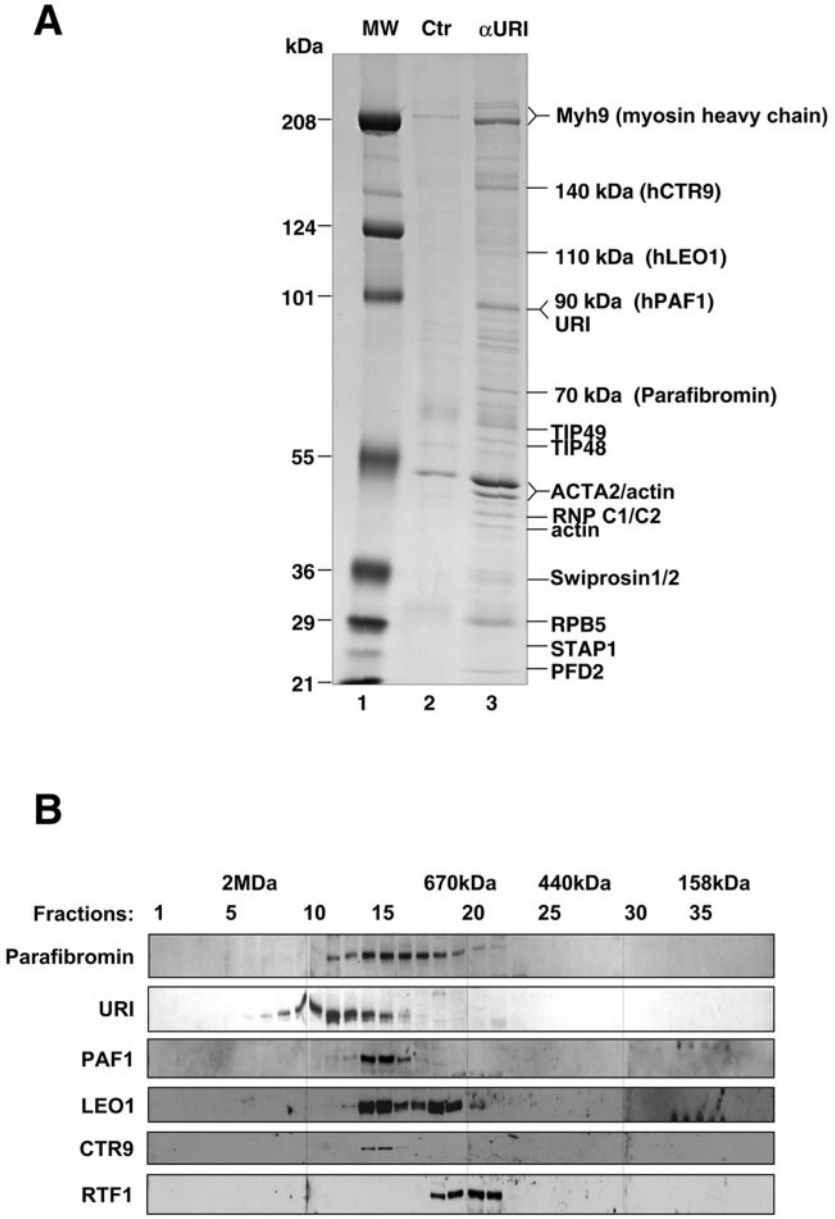

FIG. 1. Mass spectrometric identification of URI-associated proteins and gel filtration analysis (A) HEK-293 whole-cell extracts were subjected to immunoprecipitation with control mouse IgG (Ctr, lane 2) or a mix of URI MAbs 179.2.1, 179.22.34, and 179.58.2 (αURI, lane 3) and resolved by SDS-PAGE. Polypeptides that yielded unambiguous mass spectrometry spectra are indicated. MW, molecular weight standard (lane 1). (B) A whole-cell lysate of HeLa cells was fractionated on a Superose 6 gel-filtration column, and individual fractions were processed for Western blotting with antibodies to the indicated proteins.

form of CTD is detected. Therefore, we sought to determine whether parafibromin would specifically interact with one or more forms of RNAP II phosphorylated within the CTD. To this end, we sought to detect serine 2- or serine 5-phosphorylated RNAP II in anti-parafibromin(cfp) immunoprecipitates by immunoblotting with MAb specific for each phosphorylated form of RNAP II. As shown in Fig. 3C, both serine 2- and serine 5-phosphorylated forms of RNAP II specifically associate with parafibromin in vivo (lane 1). No such association was detected in control precipitates (lanes 2 and 3). The physical association of parafibromin with the aforementioned phosphorylated forms of RNAP II implies a function for this tumor suppressor in one or more steps during the transcription cycle.

A naturally occurring loss-of-function mutant of parafibromin lacks PAF1 and RNAP II binding activity. As depicted in Fig. 4A, of all of the mutations in parafibromin observed in affected families, ca. 80% arise in a so-called "mutational hotspot' in the N-terminal segment of the *HRPT2* gene product, resulting in carboxy-terminal truncations that delete more than half of the coding sequence. This suggests that the C-terminal domain of parafibromin might be important for its tumor suppressor function. Sequence analysis of parafibromin's primary

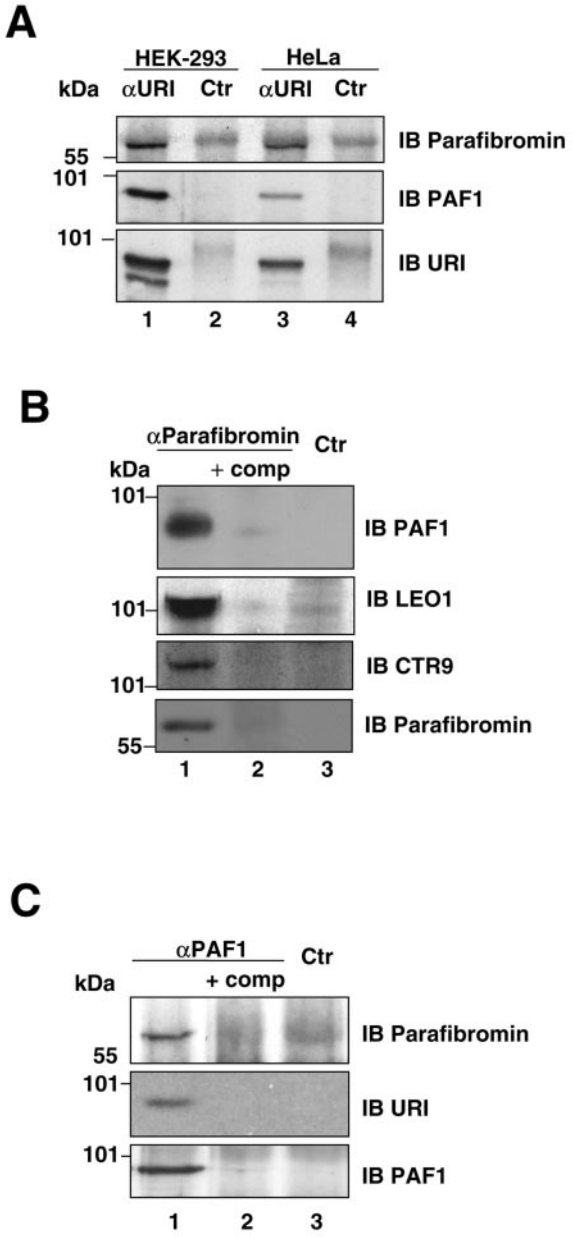

FIG. 2. URI, parafibromin, and PAF1 complex components interact in vivo. (A) HEK-293 or HeLa cell extracts were subjected to immunoprecipitation with anti-URI MAbs (lanes 1 and 3) or control mouse IgG (lanes 2 and 4) and processed for immunoblotting with anti-parafibromin(cp), anti-PAF1(cp), or anti-URI MAbs. (B) HeLa cell extracts were subjected to immunoprecipitation with anti-parafibromin(cfp) antibodies in the absence (lane 1) or presence of competing GST-parafibromin(378-531) fusion protein (lane 2). Lane 3, control rabbit IgG. Immunoprecipitates were processed for immunoblotting with anti-PAF1(cp), anti-LEO1, anti-CTR9, or anti-parafibromin(cp) antibodies. (C) HeLa cell extracts were processed as described in panel B, except that anti-PAF1(cp) antibody was used for immunoprecipitation and immunization peptide at a molar ratio of 1/1 was used as the competitor. Membranes were probed with anti-parafibromin(cp), anti-PAF1(cp), or anti-URI antibodies. All coupled immunoprecipitation-immunoblot data shown here are representative of at least three independent experiments.

structure unveiled two potential bipartite nuclear localization signals (NLS). One is located in the N terminus (amino acids 76 to 93) and another one is located in the C terminus (amino acids 393 to 410) of parafibromin (see Fig. 4A). Thus, one could imagine that loss-of-function mutations of parafibromin might impair the normal localization of parafibromin. Alternatively, such loss-of-function mutants might have lost the capacity to engage in complex formation with PAF1 and RNAP II.

To test these possibilities, we generated expression plasmids encoding N-terminally, HA-tagged parafibromin wild type, a naturally occurring truncation mutant of it lacking the carboxy-terminal 312 amino acids (referred to as R222X) and an N-terminal deletion mutant of parafibromin comprising amino acid residues 200 to 531 [referred to as parafibromin(200-531)]. Parafibromin(wt), when transiently expressed in HeLa cells, localized to the nucleus (Fig. 4B), like endogenous protein (Fig. 4C), as evidenced by indirect immunofluorescence microscopy. The R222X mutant derivative of it behaved similarly (Fig. 4B). Thus, the tumorigenic potential of at least some mutants of parafibromin lacking the C-terminal end of the protein, could not be explained by a mislocalization of the protein. The N-terminal deletion mutant parafibromin(200-531) localized in part in the cytoplasm (Fig. 4B), implying that N-terminal sequences contribute to the proper localization of the tumor suppressor protein. Indeed, when this specific parafibromin mutant was equipped with a simian virus 40-large T NLS (referred to as NLS-200-531), exclusive nuclear localization was observed (Fig. 4B). As a first assessment of whether the naturally occurring mutant R222X is impaired in PAF1 binding, we produced it and wild-type parafibromin as GST fusion proteins. Equal amounts of each protein bound to glutathione-Sepharose beads were then incubated with in vitro translated PAF1. Although GST-parafibromin(wt) fusion protein bound efficiently PAF1 (Fig. 4D, lane 3), the mutant did not (lane 4). In a converse experiment, GST-PAF1 bound in vitro-translated parafibromin wild-type but not its mutant derivative (data not shown). Therefore, a parafibromin domain, which is deleted in the majority of tumor-derived mutants, participates in binding to PAF1 in vitro.

To determine whether the same mutant species is also defective for PAF1 binding in vivo, it was produced in parallel to wild-type parafibromin in HeLa cells and analyzed for association with endogenous PAF1. As shown in Fig. 4E, HA-tagged parafibromin(wt) was detected in anti-PAF1 immunoprecipitates (lane 3) but not in control immunoprecipitates (lane 4). Importantly, HA-parafibromin(R222X) failed to coimmunoprecipitate with endogenous PAF1 (lane 5) despite the presence of similar levels of wild-type and mutant HA-parafibromin in the lysates (compare lanes 2 and 1). Similarly, parafibromin(R222X) was also defective in binding to RNAP II (Fig. 4F, lane 5), whereas wild-type protein bound efficiently to RNAP II (lane 3). Taken together, these results strongly suggest that the C-terminal segment of parafibromin is important for both PAF1 and RNAP II binding. In keeping with these findings, the N-terminal parafibromin truncation mutant (NLS-200-531) bound efficiently PAF1 and RNAP II when analyzed in parallel (Fig. 4E and F, lane 8, respectively), suggesting that this CTD contains sequences that are necessary and sufficient for PAF1 and RNAP II interactions.

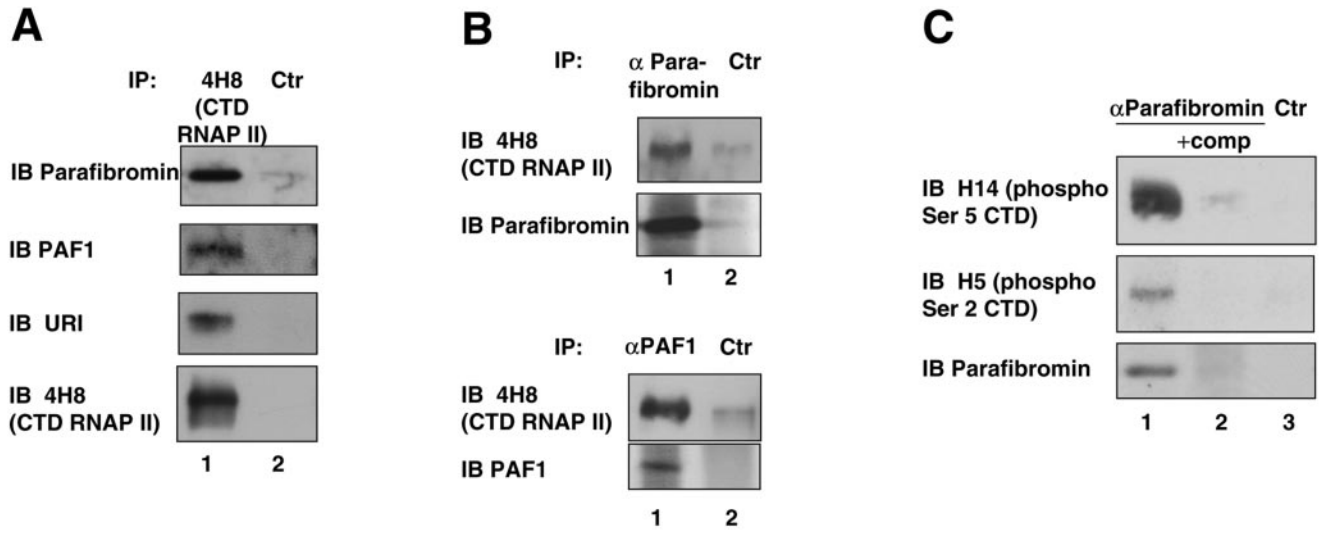

FIG. 3. Parafibromin and PAF1 interact with RNAP II. (A) HeLa cell extracts were subjected to immunoprecipitation with anti-RNAP II MAb 4H8 (lane 1) or control mouse IgG (lane 2). Immunoprecipitates were analyzed by immunoblotting with the indicated antibodies. (B) Parafibromin (upper panel) and hPAF1 (lower panel) immunoprecipitates were performed as described for Fig. 2B and C and immunoblotted with 4H8 MAb. (C) Parafibromin immunoprecipitates were immunoblotted with either anti-RNAP II MAb H14 (upper panel) or H5 (middle panel) specific for the phosphorylation of RNAP II CTD on Ser5 and Ser2, respectively, or anti-parafibromin(cp) (lower panel). As competitor, GST-parafibromin(378-531) fusion protein was used (lane 2). All coupled immunoprecipitation-immunoblot data shown here are representative of at least three independent experiments.

Parafibromin inhibits S-phase progression and facilitates cell cycle exit upon serum deprivation. Mutations in the *HRPT2* gene have recently been correlated with the lack of detectable expression of parafibromin in the corresponding parathyroid tumor tissue (36). Thus, one might argue that in tumor cells lacking parafibromin, one or more aspects of PAF1 complex function might be disrupted. In this regard, previous phenotypic analysis of yeast Paf1 complex components, including Cdc73 and Paf1, have revealed direct linkages between the presence of these proteins and proper cell cycle regulation (26). Thus, we assessed whether knockdown of parafibromin or PAF1 by RNA interference would affect cell cycle progression in mammalian cells. The siRNA oligonucleotides used in these experiments were each very efficient in downregulating parafibromin or PAF1 (Fig. 5A, lanes 3 and 2, respectively). Although treatment of HeLa cells with control siRNA did not alter the distribution of the population of cells in the cell cycle, parafibromin or PAF1 siRNA caused an increase in the fraction of cells in S phase with a concomitant reduction of cells in G_1 (Fig. 5B, upper panels). Thus, part of parafibromin's (and PAF1's) normal function may be to inhibit S-phase entry. In accord with this notion, we found that depletion of parafibromin or PAF1 by RNAi delayed cell cycle exit in response to serum withdrawal, as evidenced by the fact that a significant fraction of cells remained in the cell cycle despite the absence of serum (Fig. 5B, lower panels). Hence, a tumor suppressor that specifically interacts with PAF1 and certain modified forms of RNAP II also exhibits G_1 exit control function.

DISCUSSION

The results presented here reveal that the *HRPT2* gene product parafibromin, a tumor suppressor of as-yet-unknown function, participates in a pathway important for the coordination of various steps of the mRNA synthesis process. This conclusion is based on the findings that parafibromin directly coimmunoprecipitates with human orthologs of yeast Paf1 complex components, including PAF1, LEO1, and CTR9, that occupy a central role in the transcription elongation and RNA processing process and with specific phosphorylated forms of RNAP II that serve active roles in the temporal coordination of events of the gene expression pathway, in particular transcriptional initiation, elongation, and 3′-end processing. Similar observations have been reported by Meyerson and coworkers during revision of the present study (28).

The result that parafibromin interacts with human PAF1 complex components and with forms of RNAP II whose CTD is phosphorylated on serine 5 and serine 2 constitutes now a significant body of evidence, which, when viewed in light of the fact that parafibromin and Cdc73 share homology (albeit moderate) at the primary sequence level, argues that the tumor suppressor parafibromin is the human ortholog of Cdc73 and a component of a human PAF1 complex. The homology between parafibromin and Cdc73 is most apparent in the C-terminal part of the proteins, suggesting that this segment defines an evolutionary conserved functional domain. Indeed, this specific domain of parafibromin is necessary and sufficient for PAF1 binding and for stable association with RNAP II.

Although the structural basis for the various protein-protein interactions within these complexes are not fully understood, it would appear that the C-terminal segment of parafibromin is critical for PAF1 and RNAP II binding. The actual sequences within this ~300-residue C-terminal fragment of parafibromin required for these interactions have not yet been identified. Nonetheless, it is interesting that this specific segment of parafibromin is deleted in the majority of tumor-derived mutants.

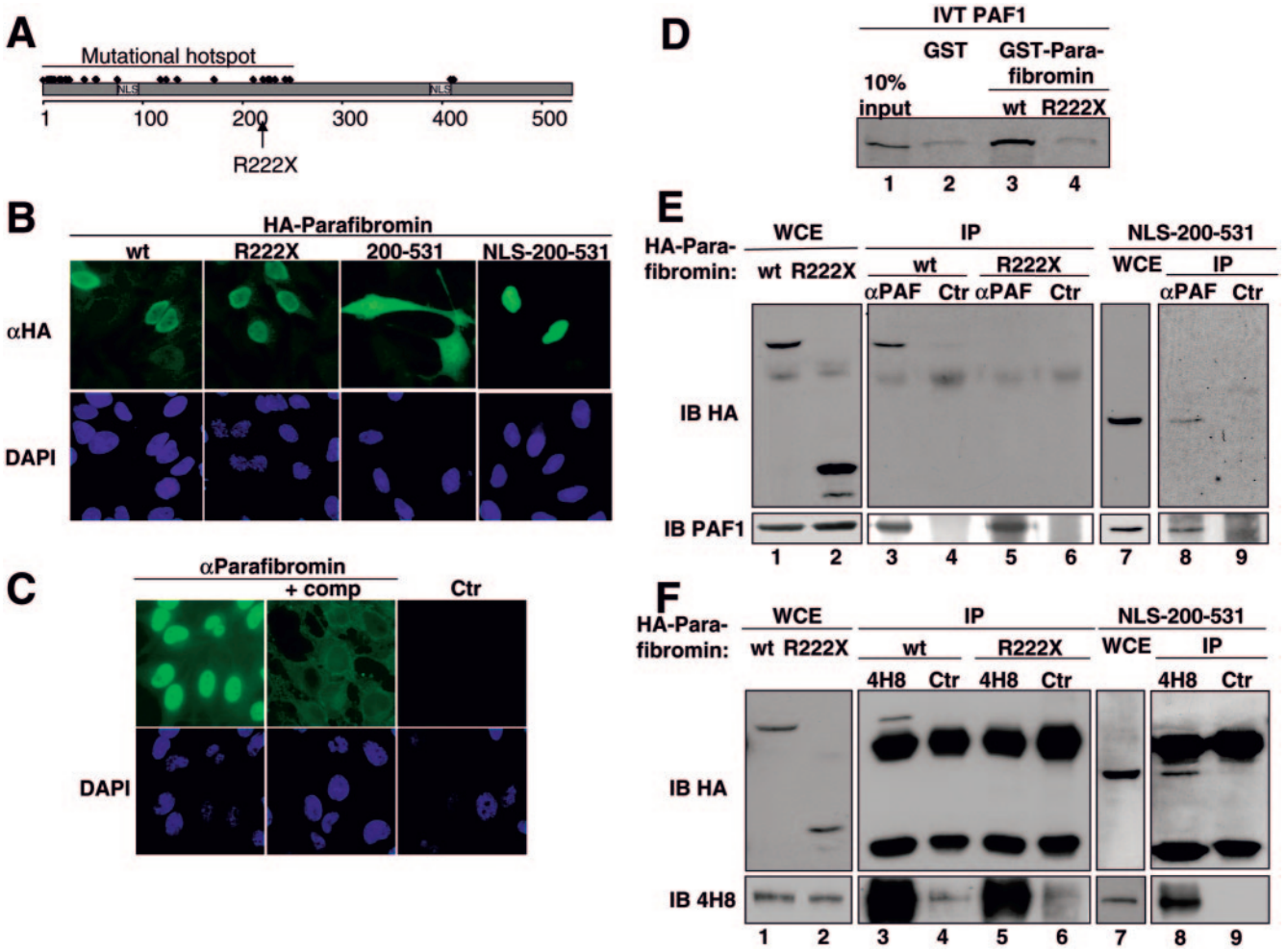

FIG. 4. A naturally occurring tumor-derived mutant of parafibromin fails to associate with PAF1 and RNAP II. (A) Schematic representation of parafibromin. Black diamonds indicate known mutations in the *HRPT2* gene. The mutation R222X used in the present study is shown. (B) HeLa cells transfected with plasmids encoding HA-tagged parafibromin(wt), R222X mutant (R222X), or parafibromin(200-531), were double stained with anti-HA MAb 12CA5 (upper panels) and DAPI and processed for indirect immunofluorescence microscopy. Note that both parafibromin(wt) and R222X mutant localize to the nucleus, whereas parafibromin(200-531) also displays a cytoplasmic localization. (C) Untransfected HeLa cells were either double stained with affinity-purified anti-parafibromin(cfp) antibody and DAPI without (left panels) or with (middle panels) prior preadsorption of antibody on glutathione-Sepharose beads loaded with GST-parafibromin(378-531) fusion protein or with secondary antibody alone (right panels). (D) HA-PAF1 was in vitro translated, incubated with indicated GST fusion proteins, and subjected to SDS-PAGE (lanes 2 to 4). Lane 1, input IVT. GST fusion proteins amounts were normalized by Coomassie blue staining prior to incubation with the IVT (data not shown). (E) HeLa cells transfected with HA-tagged parafibromin(wt), R222X mutant or parafibromin(200-531) expression plasmids were lysed, and aliquots were subjected to immunoprecipitation with either anti-PAF1(cp) (lanes 3, 5, and 8) or control rabbit IgG (lanes 4, 6, and 9) and processed for immunoblotting with anti-HA MAb 12CA5 (upper panel) or anti-PAF1(cp) antibody (lower panel). Other aliquots were directly processed for immunoblotting with indicated antibodies. (F) HeLa cells were transfected and processed as in panel E except that anti-RNAP II MAb 4H8 was used for immunoprecipitation. All coupled immunoprecipitation-immunoblot data shown here are representative of at least three independent experiments.

This finding suggests a functional relationship between these factors and enforces the impression that at least part of parafibromin's tumor suppressor function may be mediated through interactions with PAF1 and/or RNAP II.

What might be the outcome of a specific parafibromin interaction with PAF1 and RNAP II? In yeast, the Paf1 complex has been linked to various processes of the transcription cycle, including transcription initiation, elongation, and RNA processing (21, 25, 27, 33, 34). These effects of the Paf1 complex can be explained at least in part through its ability to associate with forms of RNAP II whose CTD is phosphorylated on serine 5 and serine 2 and with specific enzymatic activities. These include the Bre1 E3 ubiquitin protein ligase involved in histone H2B monoubiquitination (40) and Set1 that mediates subsequent histone H3 lysine 4 (H3-K4) trimethylation, a signature of gene activity (23). In this regard, it appears that Cdc73 binds directly to RNAP II and as such bridges Paf1 to the RNAP II holoenzyme (21, 32). Thus, one possible innate function of parafibromin within the PAF1 complex might be to contribute to transcriptional control by allowing the proper assemblage of multiprotein complexes and associated enzymatic activities involved in chromatin modification on RNAP

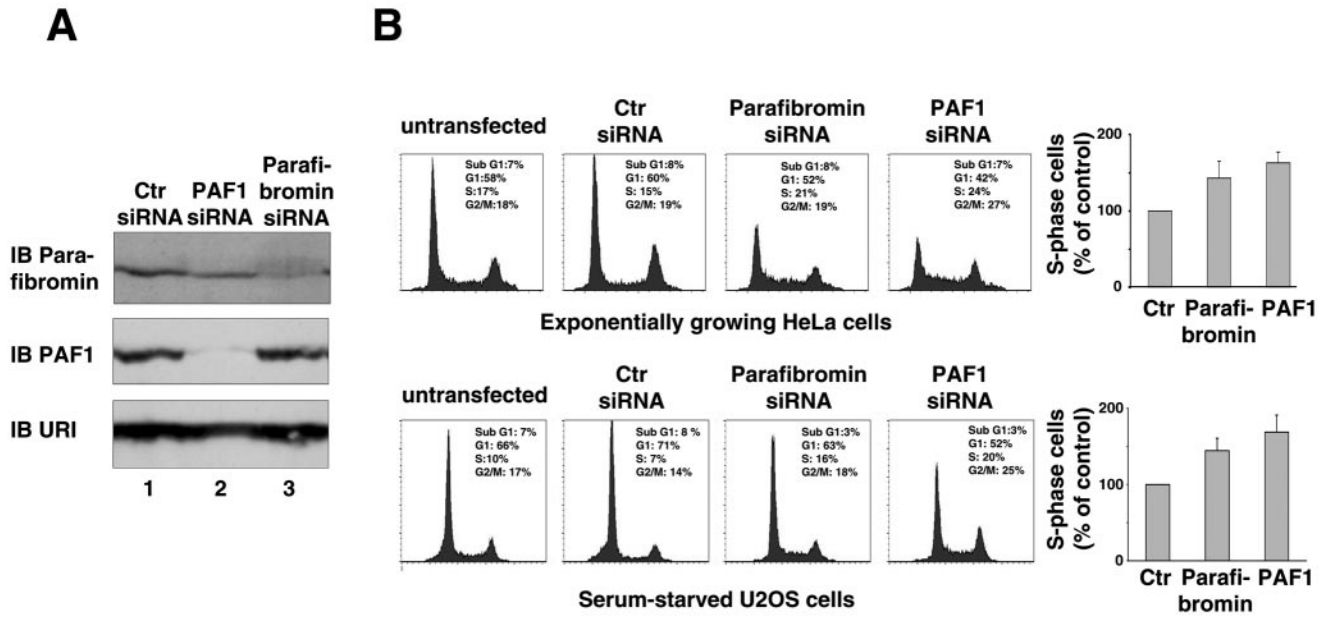

FIG. 5. siRNA-mediated knockdown of parafibromin and PAF1 induces entry into S phase. (A) HeLa cells were transfected with either siRNA specific for parafibromin or PAF1 or nonsilencing RNA (Ctr) as a control. At 48 h posttransfection, cells were harvested and processed for immunoblotting with the indicated antibodies. (B) In the upper panel, HeLa cells were transfected as described for panel A and processed for fluorescence-activated cell sorting (FACS) analysis. In the lower panel, U2-OS cells were transfected with the indicated siRNA. After 24 h, cells were serum starved for 48 h and analyzed by FACS. FACS profiles are representative of at least three independent experiments. The right panels show the numbers of S-phase cells expressed as a percentage of the control and represent the mean ± the standard error of at least three independent experiments.

II. That parafibromin is able to recruit a histone H3 lysine 4 methyltransferase would be consistent with such a proposal (28). In addition, the Paf1 complex has also been implicated in coupling transcriptional and posttranscriptional events through modulation of the levels of serine 2 phosphorylation of RNAP within CTD and poly(A) length. Thus, it is conceivable that an alternative (but not mutually exclusive) function of parafibromin might be related to the regulation of posttranscriptional events that determine the fate of a newly made mRNA. Accordingly, naturally occurring mutants of parafibromin that lack the relevant sequences involved in PAF1/RNAP II binding may therefore be defective in the above-noted functions. In this context, we note that in yeast the failure of Paf1 factors to associate with chromatin had little phenotypic consequences, implying potential additional functions of PAF1 complex components beyond transcription (21). Lastly, the role of the unconventional prefoldin subunit URI in the context of parafibromin and the PAF1 complex remains to be defined. Of interest in this regard may be the recent observation that SWR-C, a chromatin remodeling complex identified in a genetic screen for proteins involved in chromatin modification and transcriptional elongation by RNAP II and Cdc73, also displays genetic interactions with genes encoding prefoldin subunits (14). Hence, it is tempting to speculate that URI serves a chaperone function within the parafibromin-linked multiprotein complex. That URI is also a target of regulation by the TOR and phosphatidylinositol 3-kinase pathways raises the intriguing possibility that URI-parafibromin-PAF1 complexes may be transcriptional endpoints of nutritional and growth factor cues.

Although the identities of the genes regulated by parafibromin and the human PAF1 complex are not known at present, in yeast Cdc73 target genes include genes whose products participate in cell cycle control (26). Thus, parafibromin may contribute, at least in part, directly or indirectly to normal cell cycle progression. This view is consistent with the observation that experimental downregulation of parafibromin by siRNA resulted in premature entry of cells into S phase and in a failure of cells to exit the cell cycle upon serum withdrawal. Interestingly, we observed similar cell cycle effects when PAF1 was downregulated by siRNA, implying that the negative effects of parafibromin on G_1-phase progression might be linked to its functional association with PAF1. Given these results, it will be interesting to explore whether PAF1 itself has tumor suppressing activity. Irrespective of this, one could imagine that parathyroid tumorigenesis initiated by functional inactivation of the *HRPT2* gene may be a direct result of altered cell cycle control. Clearly, further study is needed to elucidate how alterations in the mRNA synthesis process caused by the functional inactivation of parafibromin translate into defects in cell cycle control and cell transformation.

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